Development of an indirect α-actinin-based immunoassay for the evaluation of protein breakdown and quality loss in fish species subjected to different chilling methods

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Summary

α-Actinin release from the myofibrillar protein fraction to the sarcoplasm can be considered as an accurate proteolysis index in seafood muscle. The main objective of the present work was to develop a specific enzyme-linked immunosorbent assay (ELISA), based on the use of a monoclonal antibody against α-actinin to evaluate the proteolysis degree in two different chilled fish species –European hake (*Merluccius merluccius)* and turbot (*Psetta maxima*)- kept under two different storage systems: flake ice and slurry ice. Comparison with sensory assessment, K value and sarcoplasmic protein profiles was carried out. A different proteolysis degree could be observed in both fish species; thus, the immunoassay evaluated has shown to be useful in monitoring the protein degradation events in hake muscle especially under flake ice storage. In the case of turbot, very low proteolysis development could be obtained, so that the assay could not be suitable for assessing quality changes. A different break point of inmunoassay values for each type of fish species is suggested to be necessary.

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Keywords: Hake, turbot, refrigeration, liquid ice, α-actinin, ELISA, quality

Running title: alpha-actinin assessment in chilled fish by specific ELISA

INTRODUCTION

The post-mortem tenderization of fish muscle is one of the major problems related to the loss of freshness and quality in chilled seafood products. One of the main causes of such tenderization is the breakdown of Z-line structures of the muscle fibber (Ando *et al*., 1991). The principal protein of Z-line is α-actinin. In fish muscle, α-actinin (100 kDa, p*I* 5.6) (Papa *et al*., 1995), represents 2 % of total myofibrillar protein content (Takahasi & Hatori, 1992). This myofibrillar protein has been reported to be involved in the anchorage of end-to-end actin filaments with opposite polarity to the Z-line between adjacent sarcomeres. In addition, this protein is bound to elastic titin filaments in the Z-line by N-terminal part and M-line-associated proteins in the middle of the sarcomere by the C-terminal end of the same actinin molecule (Trinick, 1991; Small *et al*., 1992).

α-Actinin has also been reported to play a key role in postmortem changes affecting the Z-line structure (Astier *et al*., 1991; Seki & Tsuchiya, 1991; Papa *et al*., 1996). Thus, previous reports have proposed that the release of α-actinin from the myofibrillar protein fraction depends on different proteolytic mechanisms, such as the activity of endogenous proteases like calpains and cathepsins (Goll *et al*., 1992; Taylor *et al*., 1995; Aoki *et al*., 2000; Lamare *et al*., 2002; Verrez-Bagnis *et al*., 2002; Ladrat *et al*., 2003; Delbarre-Ladrat *et al.,* 2004a, 2004b).

With a view to slowing down such fish deterioration, different methods during the chilling storage (Ashie *et al*., 1996) such as traditional flake ice (Nunes *et al*., 1992), refrigerated seawater (Kraus, 1992) and the use of chemical additives (Ponce de León *et al*., 1993; Hwang & Regestein, 1995) have been applied. Recently, slurry ice (an ice-water suspension at subzero temperature) –also known as fluid ice, flow ice, slush ice or liquid ice– has been reported to be a promising technique for the preservation of aquatic food products (Chapman, 1990; Harada, 1991; Huidobro *et al.*, 2002). In previous

reports our group has studied the quality loss of different seafood species when refrigerated under flake and slurry ice conditions (Losada *et al*., 2004; Piñeiro *et al*., 2004, Aubourg *et al*., 2005; Rodriguez *et al*., 2006).

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Currently, the methods employed for monitoring changes associated with quality loss in fish can be classified as sensory, physical, physico-chemical, chemical and microbiological (Olafsdóttir *et al*., 1997). Among these, the study of the release of α-actinin to the sarcoplasmic fraction has been considered as a useful procedure for evaluating the rate of proteolysis in seafood, being this protein proposed as a potential biomarker of quality and freshness in chilled fish (Bandman & Zdanis, 1988; Tsuchiya *et al*., 1992; Papa *et al*., 1995; Papa *et al*., 1996; Verrez-Bagnis *et al*., 1999; Morzel *et al*., 2000; Delbarre-Ladrat *et al*., 2004b) even in the earliest postmortem steps.To our knowledge, no previous method based on the detection of α-actinin by means of a specific enzyme-linked immunosorbent assay (ELISA) has been developed up to now with the purpose of evaluating the rate of proteolysis in chilled fish species.

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In a first attempt (Carrera et al., 2004), our group applied this immunoassay procedure to evaluate the α-actinin release in three different fish species (hake, turbot and horse mackerel) during their chilled storage under flake ice conditions. As a result, species showing a low fat content (hake and turbot) provided promising correlation values between the optical densities derived from the ELISA assay and the traditional quality loss indices. The present work was focused on both lean species, taking into account the effect that differences such as the nature of the fish species and size may undergo on autolytic degradation and quality loss (Huss, 1999, Dalgaard, 2000).

The main objective of this study was to assess the evolution of the freshness loss in hake and turbot during the chilled storage under two different systems (flake ice and slurry ice) by the above mentioned ELISA assay. Comparison with results obtained

from sarcoplasmic protein electrophoretic profiles, nucleotide degradation and sensory assessment was carried out.

MATERIALS AND METHODS

Fish material, processing and sampling.

European hake (*Merluccius merluccius*) specimens were caught off the Atlantic coast of North Western Spain and kept in flake ice until they arrived to our laboratory (six hours later). Farmed turbot (*Psetta maxima*) specimens were obtained from Stolt Sea Farm, S.A. (Carnota, Spain) and kept on flake ice for 6 hours until they arrived to our laboratory. For both fish species, individual specimens were divided into two batches, being one of them reserved to the flake ice treatment, while the other corresponded to slurry ice process. Fish specimens (not headed, not gutted) were placed under both icing conditions inside an isothermal room at 2 ºC. Fish samples from both icing conditions were taken for analysis on days 2, 5, 8, 12, 15 and 19 for hake, and on days 2, 5, 9, 14, 19, 22, 26, 29, 33, 36 and 40 for turbot. Starting material from both fish species was also studied. For each species and each chilling systems, three different groups (n=3) were studied separately during the whole experimental period.

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Refrigeration systems.

In this work, a slurry ice prototype (FLO-ICE^{TM}, Kinarca S.A.U., Vigo, Spain) was used. The composition of the slurry-ice binary mixture, prepared from filtered seawater, was 40 % ice/60 % water, being the temperature adjusted to -1.5 ºC. Flake ice was prepared with an Icematic F100 Compact device (CASTELMAC SPA, Castelfranco, Italy). The temperature of the flake ice was -0.5 ºC. The fish specimens were

surrounded by flake ice or slurry ice at a fish/ice ratio of 1/1. When required, both ices were renewed.

Sensory analysis.

Sensory analysis was conducted by a panel consisting of five experienced judges, who based appraisals according to guidelines concerning fresh and refrigerated fish (Council Directive 91/493/EEC 1991; Rodríguez *et al*., 2003). Four categories were ranked: highest quality (E), good quality (A), fair quality (B) and unacceptable quality (C). Sensory assessment of the fish included the examination of the following parameters: skin, external odour, gills, consistency and flesh odour. Once fish specimens had been subjected to sensory analyses, the white muscle was separated and used for biochemical analyses.

Nucleotide degradation analysis.

Analysis of the autolytic nucleotide degradation in fish muscle was carried out by HPLC analysis according to the method of Ryder (1985). The K value was calculated according to the following concentration ratio: K value (%) = 100 x (hypoxanthine + inosine) / (adenosine triphosphate + adenosine diphosphate + adenosine monophosphate + inosine monophosphate + inosine + hypoxanthine).

Solubilization of sarcoplasmic protein fraction.

Sarcoplasmic protein extracts from fish muscle were prepared in a low-ionic-strength buffer composed of 10 mM Tris-HCl, pH 7.2, + 50 mM PMSF (pentamethyl sulphonic acid) as previously described (Piñeiro *et al*., 1998). All extracts were maintained at –80 ºC until analysis. Protein concentrations in the extracts were determined by means of the

protein microassay method (Bio-Rad Laboratories Inc. Hercules, CA, USA). A standard curve constructed from bovine serum albumin (BSA) was used as reference.

SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoretic analyses of the sarcoplasmic protein fraction from fish muscle were carried out in commercial horizontal SDS-PAGE gels (245x110x1mm Excel-Gel SDS Homogeneous 15 %, Amersham Biosciences, Uppsala, Sweden). Protein bands were visualised by silver staining as previously described (Piñeiro *et al*., 1998).

Enzyme-linked immunosorbent assay (ELISA)

α-Actinin content in the sarcoplasmic protein extracts from fish muscle was determined by an indirect ELISA. Briefly, 96-well high-binding plates (Costar Corning Incorporated, NY, USA) were coated with aliquots from each sarcoplasmic protein extract (20 µg/mL / 50 µL), incubated at 4 ºC overnight, blocked by addition of PBS/1 % BSA solution for 2 h at 37 ºC and washed three times with PBS/0.1 % Tween 20. Then, wells were incubated with 50 µL of mouse monoclonal anti-α-actinin sarcomeric antibody (MAb) (diluted 1:500 in PBS/0.1% BSA) (Sigma, St Louis, MO, USA) during 1.5 h at 37 ºC and washed again. Bound antibodies were detected using 50 µl/per well of horseradish peroxidase (HRP)-labeled goat anti-mouse Igs (diluted 1:1000 in PBS/0.1 % BSA) (Sigma). After washing, the colorimetric reaction was developed with the addition of the substrate *ortho*-phenylene-diamine (OPD, Sigma) according to manufacturer's instruction and stopped by the addition of 3 M sulphuric acid. Finally, optical density (OD) at 492 nm was measured by means of an ELISA Microplate Reader (Labsystems iEMS Reader MF, Molecular Devices Co, Sunnyvale, CA, USA). Three replicate wells per sample and a negative-control without primary antibody were

considered in all cases. The OD value obtained for each extract was corrected by subtracting the OD value determined in negative samples.

The optimised method was validated using calibration standards at 1, 5, 10, 40, 60, 80, 100 μg/mL per well into PBS/0.1 % BSA of a commercial and purified α-actinin from chicken gizzard (Sigma).

In the absence of α-actinin purified from the fish species, the specificity of sarcomeric anti-α-actinin antibody was tested previously by western blot assay (Rybicki & von Wechmar, 1982). Sarcoplasmic protein electrophoretic profiles from chilled hake and turbot were studied by SDS-PAGE. For it, homogeneous vertical gels (10 %T and 3 %C) including 0.1 M Tris-0.1 M Tricine-0.1 % SDS pH 8.25 and 0.2 M Tris pH 8.9, as cathode and anode solutions, respectively, were employed. When the electrophoresis development was accomplished, gels were transferred to Hybond-P PVDF membranes (Amercham Biosciences) in a Mini-Trans-Blot-Cell (Bio-Rad Laboratories, Inc. Hercules, CA, USA), incubated and stained according to Rybicki and von Wechmar (1982).

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RESULTS

Fish freshness as determined by sensory analysis.

European hake specimens stored in slurry ice maintained good quality up to day 8, while hake specimens stored in flake ice exhibited good quality only up to day 2 (Table 1). In the case of turbot, the specimens chilled in slurry ice showed good quality up to day 22, while the counterpart specimens stored in flake ice did so only until day 14. A higher shelf-life time was obtained by employing slurry ice conditions for both hake and turbot when compared to their counterparts stored under flake ice. Comparison between the sensory acceptance scores of both species showed a marked faster quality loss for

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hake than for turbot. **Evaluation of protein degradation by monitoring the electrophoretic profiles of sarcoplasmic proteins.**

In the electrophoretic protein profiles obtained for hake (Figure 1*i*), a marked increase in the concentration of two protein bands of 23 and 24.5 kDa respectively was observed after 5 days of storage for the batch chilled in flake ice; these bands were subsequently degraded by day 15. However, for the hake batch stored in slurry ice, the presence of such protein bands increased only after day 12.

In turbot stored in slurry ice (Figure 1.*ii*) the sarcoplasmic protein profiles did not reveal changes in protein bands corresponding to molecular weights in the range of 87 to 94 kDa. However, when the batch chilled under flake ice is considered, such band range was found markedly modified as a result of fish damage, being visualised with great difficulty.

Previous reports have proposed certain soluble polypeptides as qualitative biomarkers of fish spoilage or freshness (Papa *et al*., 1996; Verrez-Bagnis *et al*., 1999; Morzel *et al*., 2000). These reports agree with the above mentioned changes in protein bands concerning Figure 1.

Evaluation of fish freshness by nucleotide analysis.

Assessment of nucleotide degradation was carried out by means of the K value (Figure 2, bars). In global terms, both fish species provided a different evolution. Thus, hake showed a progressive increase throughout the experiment, being in all cases the K index under 60% value. However, turbot stored under flake ice provided a logarithmic pattern with time, so that a sharp increase was observed in the 0-14 days period, that was followed by no changes; values were above 60% in the 14-40 days period in all cases.

Turbot stored under slurry ice showed a sharp increase till the end of the storage, when scores attained the 60% value.

As expected from the sensory results, and according to our previous results (Rodriguez *et al*., 2003 and Losada *et al*., 2004), storage in flake ice implied significantly higher ($p<0.05$) K values than storage in slurry ice. In the case of European hake specimens, day-to-day comparison revealed significantly ($p<0.05$) higher K values on days 12, 15 and 19 for the batch stored in traditional flake ice than for the counterpart specimens stored in slurry ice. Moreover, turbot specimens stored in flake ice showed a higher ($p<0.05$) K value development than the counterparts stored under slurry ice.

From the actual results it can be concluded that the application of slurry ice slowed down the nucleotide degradation in hake and turbot although the K value showed a very different evolution in both species, so that this quality index cannot be defined as a general parameter to evaluate the freshness in fish species.

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Development of an indirect ELISA assay based on α–actinin assessment to evaluate fish freshness.

An indirect ELISA assay employing a commercial α-actinin mouse MAb, was chosen in order to estimate the proteolysis degree concerning the α-actinin release in both chilled hake and turbot species. The specificity of the commercial α-actinin mouse MAb, was proved by the western blot procedure described in the Materials and Methods section. Figure 3 shows the single stained band obtained from α–actinin control and turbot sarcoplasmic profiles after 36 day of storage under flake and slurry ice; profile comparison showed the presence in both chilled turbots of a single fragment with similar molecular weight (100 kDa) than in the α-actinin control.

The α-actinin immunoassay was validated by employing calibration standards at 1, 5, 10, 40, 60, 80 and 100 μg/mL, prepared using a commercial α-actinin standard from chicken gizzard. Figure 4 shows the standard dependent-dose curve for pure α-actinin at different concentrations and its corresponding regression equation. The mean regression coefficient for analysis was r^2 = 0.9659. Besides, the non-specific binding of the peroxidase-labeled second antibody was tested by means of a negative control without primary antibody.

Figure 2 (lines) shows the results obtained by means of the referred ELISA method aimed to detect the α–actinin release in the sarcoplasmic protein extracts obtained from the muscle of fish specimens (hake, Figure 2i; turbot, Figure 2ii) along storage either in flake ice or slurry ice. As it can be seen, a fair correlation between the proposed ELISA method (lines) and the K value (bars) tendency during storage was observed for both species in the case of flake ice storage, showing a progressive increase for hake and a logarithmic one for turbot, accordingly to the K value evolution. However slurry ice conditions did not provide ($p>0.05$) changes in the OD values throughout the experiment, so that a good agreement with the K value was not obtained. The OD values obtained were found to be highly species-dependent, being markedly bigger in the case of hake (Figure 2i lines). Thus, low OD values (less than 0.2 units) obtained for turbot (Figure 2ii lines) are not included in the linear range of alpha-actinin calibration curve.

Specimens stored under flake ice conditions showed a higher OD values ($p<0.05$) than their counterpart specimens stored under slurry ice conditions. Thus, higher ($p<0.05$) OD values were obtained in the 15-19 day and 19-40 day periods for hake and turbot, respectively.

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DISCUSSION

Muscle proteolysis was evaluated in the postmortem stage of two different fish species stored under two different chilling systems (flake and slurry ices) by means of the α-actinin release from the Z-line along storage time and compared to sensory acceptance, electrophoretic profiles of sarcoplasmic proteins and nucleotide degradation index (K value). In the present work, a specific ELISA procedure was developped, to our knowledge for the first time, being its purpose the assessment of the α-actinin release and correlate it with the quality loss. According to the results obtained in this study, the differences observed in the proteolysis degree, directly depend on the nature and type of fish species considered, a result that agrees with previously published reports (Whittle *et al.,* 1990; Love, 1997).

Specimens of European hake stored in flake ice showed a marked increase in the OD value after day 8, according to the K value assessment, and showing to be unacceptable from the sensory analysis at this time. By contrast, specimens stored in slurry did not provide OD values differences throughout the experiment, according to a longer shelf-life time and a lower K value increase with time.

Concerning turbot specimens, the OD values obtained are in all cases below 0.2 units. According to the α-actinin calibration curve, such values would not be included in the linear range; thus, this specific immunoassay could not be suitable to evaluate the proteolysis degree in turbot species. A similar negative conclusion has previously described (Verrez-Bagnis *et al.*, 1999) when trying to assess another myofibrillar protein (desmin) as proteolysis biomarker. When compared to their counterpart profiles from flake ice system, protein profiles obtained from turbot stored in slurry ice revealed a higher intensity of proteins with molecular weight higher than 94 kDa from the first day of refrigeration up to the end of the experiment. Such protein bands could

correspond to high-molecular weight myofibrilar proteins such as α-actinin, which would be better stabilized in fish stored under the slurry ice mixture.

In summary, the immunoassay results obtained show that the refrigeration of fish using slurry ice slows down relevant deteriorative mechanisms, as it was determined by biochemical and sensory analyses. In agreement to the results obtained in this study, hake exhibited a higher protein degradation rate than turbot, since the OD values determined by means of the immunoassay were ten times higher than in the case of turbot. These results would suggest the need of defining a different break point of OD values for each type of fish species and would imply a species dependent mechanism for the alpha-actinin release from the Z-line (Papa *et al.*, 1997). In this sense, the release of α-actinin from the myofibrilar fraction may be a useful index of proteolysis.

Acknowledgements

The authors wish to thank KINARCA S.A.U. for providing the slurry ice equipment. This work was supported by two projects granted by the Ministerio de Educación y Ciencia (Project AGL2000-0440-P4-02) and Xunta de Galicia (Project PGIDTI01MAR40202PR). The authors also thank Mrs. Lorena Barros, Mr. Marcos Trigo and Mr. José M. Antonio for their excellent technical assistance.

REFERENCES

Ando, M., Toyohara, H., Shimizu, Y. & Sakaguski, M. (1991). Post-mortem tenderisation of rainbow trout (*Oncorhyncus mykiss*) muscle caused by gradual disintegration of the extracellular matrix structure. *Journal of the Science of Food and Agriculture,* **55**, 589-597.

Aoki, T., Yamashita, T. & Ueno, R. (2000). Distribution of cathepsins in red and white muscles among fish species. *Fisheries Science,* **66**, 776-782.

Ashie, I., Smith, J. & Simpson, B. (1996). Spoilage and shelf-life extension of fresh fish and shellfish. *Critical Reviews in Food Science & Nutrition*, **36**, 87-121.

Astier, C., Labbe, J., Roustan, C. & Benyamin, Y. (1991). Sarcomeric disorganization in post-mortem fish muscles. *Comparative Biochemical & Physiology Part B*, **100**, 459-465.

Aubourg, S., Piñeiro, C., Gallardo, J.M. & Barros-Velázquez, J. (2005). Biochemical changes and quality loss during chilled storage of turbot (*Psetta maxima*). *Food Chemistry,* **90**, 445-452.

Bandman, E. & Zdanis, D. (1988). An immunological method to assess protein degradation in post-mortem muscle. *Meat Science*, **22**, 1-19.

Carrera, M., Losada, V., Piñeiro, C., Barros, L., Gallardo, J.M., Barros-Velázquez, J. & Aubourg, S.P. (2004). Evaluation of seafood proteolysis by immunological techniques: α-actinin as a biomarker of shelf-life in chilled products. In: *Proceedings of the 34th WEFTA Conference*. Pp-200-203. ISBN 3-00-013931-1. Hamburg, Germany.

Chapman, L. (1990). Making the grade. Ice slurries get top marks for quality products. *Australian Fisheries*, **7**, 16-19.

Council Directive 91/493/EEC of 22 July (1991). Laying down the health conditions for the production and the placing on the market of fishery products. *Official Journal (L),* **268**. 24/09/1991. Pp. 0015–0034.

Dalgaard P. 2000. Freshness, Quality and Safety in Seafood. Technical Manual prepared for FLAIR-FLOW EUROPE. http://www.exp.ie/flair.html. Delbarre-Ladrat, C., Verrez-Bagnis, V., Noël, J. & Fleurence J. (2004a). Proteolytic potential in white muscle of sea bass *(Dicentrarchus labrax L.)* during post mortem storage on ice: time-dependent changes in the activity of the components of the calpain system. *Food Chemistry*, **84**, 441-446.

Delbarre-Ladrat, C., Verrez-Bagnis, V., Noël, J. & Fleurence, J. (2004b). Relative contribution of calpain and cathepsins to protein degradation in muscle of sea bass (*Dicentrarchus labrax* L.). *Food Chemistry*, **88**, 389-395.

Goll, D., Taylor, R., Christiansen, J. & Thompson, V. (1992). Role of proteinases and protein turnover in muscle growth and meat quality. In: *Proc. 44th Annu. Recip. Meat Conf. National Live Stock and Meat Board*. Pp. 25-36. Chicago, IL, USA.

Harada, K. (1991). How to handle Albacore. *Australian Fisheries*, **2**, 28-30.

Huidobro, A., López-Caballero, M. & Mendes, R. (2002). Onboard processing of deepwater pink shrimp (*Parapenaeus longirostris*) with liquid ice: efect on quality. *Zeitschrift für Lebensmittel Untersuchung und Forschung*, **214**, 469-475.

Hwang, K. & Regenstein, J. (1995). Hydrolysis and oxidation of mackerel (*Scomber scombrus*) mince lipids with NaOCl and NaF treatments. *Journal of Aquatic Food Products Technology*, **4**, 19-30.

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Huss H.H. (1999). Quality and quality changes in fresh fish. FAO Fisheries Technical Paper 348. **Food And Agriculture Organization Of The United Nations.** Rome**, Italy.**Kraus, L. (1992). Refrigerated sea water treatment of herring and mackerel for human consumption. In: *Pelagic fish. The resource and its exploitation* (edited by Burt, J.R., Hardy, R. & Whittle, K.J.). Pp. 73-81. Fishing News Books, Aberdeen, Scotland, UK.

Ladrat, C., Verrez-Bagnis, V., Noël, J. & Fleurence, J. (2003). *In vitro* proteolysis of myofibrillar and sarcoplasmic proteins of white muscle of sea bass (*Dicercharchus labrax L.*): effects of cathepsins B, D and L. *Food Chemistry*, **81**, 517-525.

Lamare, M., Taylor, R., Farout, L., Briand, Y. & Briand, M. (2002). Changes in proteasome activity during postmortem aging of bovine muscle. *Meat Science*, **61**, 199-204.

Losada, V., Piñeiro, C., Barros-Velázquez, J. & Aubourg, S. (2004). Effect of slurry ice on chemical changes related to quality loss during European hake (*Merluccius merluccius*) chilled storage. *European Food Research and Technology*, **219**, 27-31.

Love, R. (1997). Biochemical dynamics and the quality of fresh and frozen fish. In: *Fish processing technology* (edited by Hall, G.). Pp. 1-31. Blackie, London, UK.

Morzel, M., Verrez-Bagnis, V., Arendt, E. & Fleurence, J. (2000). Use of two-dimensional electrophoresis to evaluate proteolysis in salmon (*Salmo salar*) muscle as affected by a lactic fermentation. *Journal of Agricultural and Food Chemistry*, **48**, 239-244.

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Nunes, M., Batista, I. & Morâo de Campos, R. (1992). Physical, chemical and sensory analysis of sardine (*Sardina pilchardus*) stored in ice. *Journal of the Science of Food and Agriculture,* **59**, 37-43.

Olafsdóttir, G., Martinsdóttir, E., Oehlenschläger, J., Dalgaard, P., Jensen, B., Undeland, I., Mackie, I., Henehan, G., Nielsen, J., & Nilsen, H. (1997). Methods to evaluate fish freshness in research and industry. *Trends in Food Science and Technology,* **8**, 258-265.

Papa, I., Méjean, C., Lebart, M., Astier, C., Roustan, C., Benyamin, Y., Alvarez, C., Verrez-Bagnis, V. & Fleurence, J. (1995). Isolation and properties of white skeletal muscle alpha-actinin from sea-trout (*Salmo trutta*) and bass (*Dicentrarchus labrax*). *Comparative Biochemistry & Physiology Part B*, **112**, 271-282.

Papa, I., Alvarez, C., Verrez-Bagnis, V., Fleurence, J. & Benyamin, Y. (1996). Postmortem release of fish white muscle alpha-actinin as a marker of disorganisation. *Journal of the Science of Food and Agriculture*, **72**, 63-70.

Papa, I., Alvarez, C., Verrez-Bagnis, V., Fleurence, J. & Benjamin, Y. (1997). Evidence for time dependent alpha-actinin delocalisation and proteolysis from *post mortem* fish white muscle (*D. labrax* and *S. trutta*). In: *Seafood from producer to consumer, integrated approach to quality* (edited by Luten J., Borrensen T. & Oehlenschläger J.). Pp. 247-251. Elsevier Science. Amsterdam, Netherlands.

Piñeiro, C., Barros-Velázquez, J., Sotelo, C., Pérez-Martín, R. & Gallardo, J.M. (1998). Two-dimensional electrophoretic study of the water-soluble protein fraction in white muscle of gadoid fish species. *Journal of Agricultural and Food Chemistry,* **46**, 3991-3997.

Piñeiro, C., Barros-Velázquez, J. & Aubourg S. (2004). Effects of newer slurry ice systems on the quality of aquatic food products: a comparative review versus flake-ice chilling methods. *Trends in Food Science & Technology*, **15**, 575-582.

Ponce de León, S., Inoue, N. & Shinano, H. (1993). Effect of acetic and citric acids on the growth and activity (VB-N) of *Pseumonas* sp. and *Moraxella* sp. *Bulletin of Faculty of Fish Hokkaido University*, **44**, 80-85.

Rodríguez, O., Barros-Velázquez, J., Ojea, A., Piñeiro, C. & Aubourg, S. (2003). Evaluation of sensory and microbiological changes and identification of proteolytic bacteria during the iced storage of turbot (*Psetta maxima*). *Journal of Food Science,* **68**, 2764-2771.

Rodríguez, O., Barros-Velázquez, J., Piñeiro, C., Gallardo, J.M. & Aubourg, S. (2006). Effects of storage in slurry ice on the microbial, chemical and sensory quality on the shelf life of turbot (*Psetta maxima*). *Food Chemistry*, **95**, 270-278.

Ryder, J. (1985). Determination of adenosine triphosphate and its breakdown products in fish muscle by high performance liquid chromatography. *Journal of Agricultural and Food Chemistry,* **33**, 678-680.

Rybicki, E.P. & von Wechmar, M.B. (1982). Enzyme-assisted immune detection of plant virus proteins electroblotted onto nitrocellulose paper. *Journal of Virological Methods*, **5**, 267-278.

Seki, N. & Tsuchiya, H. (1991). Extensive changes during storage in carp myofibrillar proteins in relation to fragmentation. *Bulletin of Nippon Suisan Gakkaishi,* **57**, 927-933.

Small, J.V., Fürst D.O. & Thornell, L.E (1992). The cytoskeletal lattice of muscle cells. *European Journal of Biochemistry,* **208**, 559-572.

Takahashi, K. & Hatori, A. (1992). Alpha-actinin is a component of the Z-filament, a structural backbone of skeletal muscle Z-discs. *Journal of Biochemistry,* **111,** 291-295.

Taylor, R., Geesink, G., Thompson, V., Koohmaraie, M. & Goll, D. (1995). Is Z-disk degradation responsible for post-mortem tenderization? J*ournal of Animal Science,* **73**, 1351-1367.

Trinick, J. (1991). Elastic filaments and gigant proteins in muscle. *Current Opinion on Cell Biology,* **3**, 112-119.

Tsuchiya, H., Kita, S. & Seki, N. (1992). Postmortem changes in α-actin and connectin in carp and rainbow trout muscles. *Bulletin of Nippon Suisan Gakkaishi,* **58**, 793-798.

Verrez-Bagnis, V., Noel, J., Sautereau, C. & Fleurence, J, (1999). Desmin degregation in postmortem fish muscle. *Journal of Food Science,* **64**, 240-242.

Verrez-Bagnis, V., Ladrat, C., Noelle. J. & Fleurence, J. (2002). *In vitro* proteolysis of myofibrillar and sarcoplasmic proteins of European Sea Bass (*Dicentrachus labrax* L) by an endogenous m-calpain. *Journal of the Science of Food and Agriculture,* **82,** 1256-1262.

Whittle, K., Hardy, R. & Hobbs G. (1990). Chilled fish and fishery products. In:. *Chilled foods: the state of the art*. (edited by Gormley T.). Pp. 87-116. Elsevier, New York, USA.

1 **Table 1.** Summary of sensory acceptance during chilled storage of both fish species

Ice treatment	Hake		Turbot	
	A[a]	C[b]	A[a]	C[b]
Slurry ice	8	15	22	29
Flake ice	2	8	14	19

2 Freshness categories: A (good) and C (unacceptable). [a]Last time (days) that "A" quality

3 was observed. [b]First time (days) that "C" quality was observed.

4

Figure captions

Figure 1: Electrophoretic profiles obtained in 15 % ExcelGel homogeneous SDS-PAGE from sarcoplasmic proteins of *i*) hake and *ii*) turbot. The different lines include a low-molecular weight standard (st: 14-94 kDa) and fish samples corresponding to the different storage times (days) for both chilling conditions. Arrows indicate the most remarkable changes detected in certain protein bands during storage.

Figure 2: Comparison between the K value and the quantitative immunoassay based on α–actinin assessment during chilled storage of: *i*) hake and *ii*) turbot . Bars indicate the K values (Y_1 axis) obtained for the slurry ice batch (white bars) and flake ice batch (black bars). Lines indicate the OD values obtained with the ELISA method for slurry ice (discontinuous line) or flake ice (solid line) conditions. Determination of α-actinin content in the sarcoplasmic protein extracts was carried out in terms of optical density (OD) at 492 nm (Y_2 axis).

Figure 3: Western blot using commercial specific mouse α-actinin MAb against chicken α-actinin as control (A), and sarcoplasmic protein extracts from turbot chilled until day 36 on slurry and flake ices.

Figure 4: α-Actinin ELISA standard curve. Absorbance values of triplicate determinations (n=3) are shown, being the standard deviation expressed by bars.

Figure 1

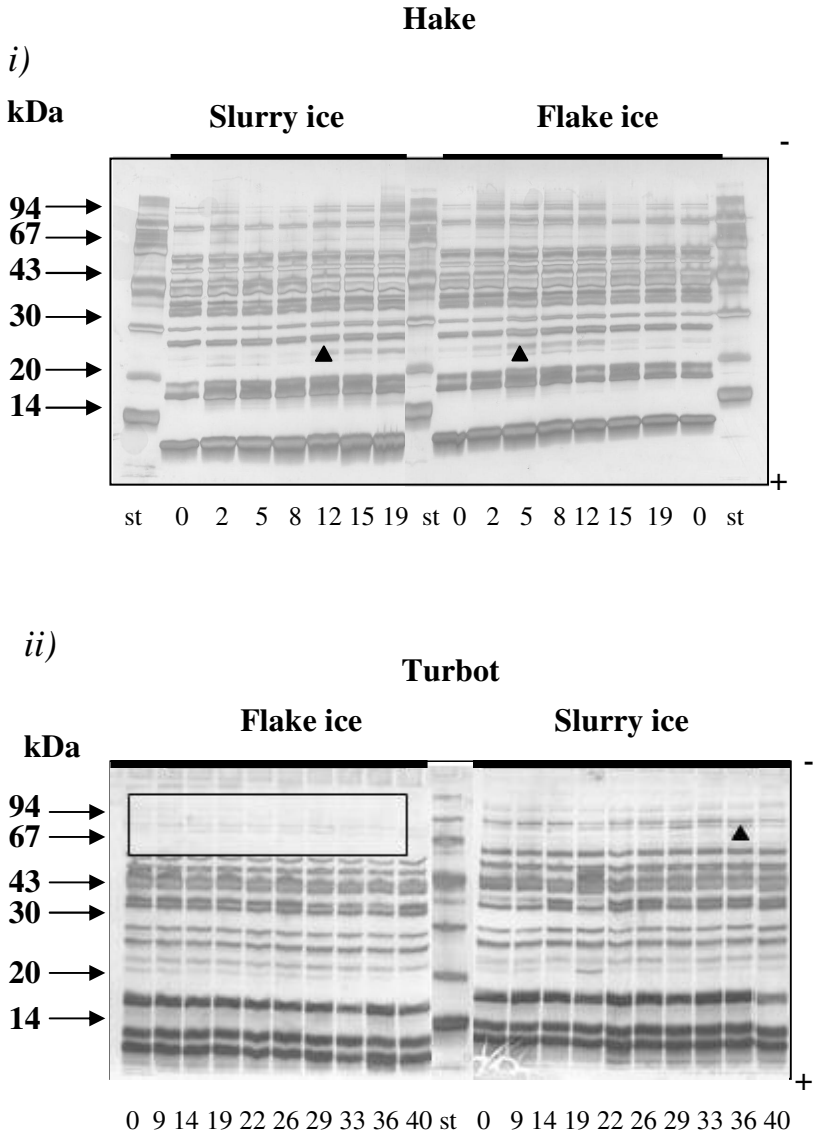

Hake
i)
kDa
Slurry ice
Flake ice
-
94
67
43
30
20
14
+
st 0 2 5 8 12 15 19 st 0 2 5 8 12 15 19 0 st
ii)
Turbot
kDa
Flake ice
Slurry ice
-
94
67
43
30
20
14
+
0 9 14 19 22 26 29 33 36 40 st 0 9 14 19 22 26 29 33 36 40

Figure *2i*

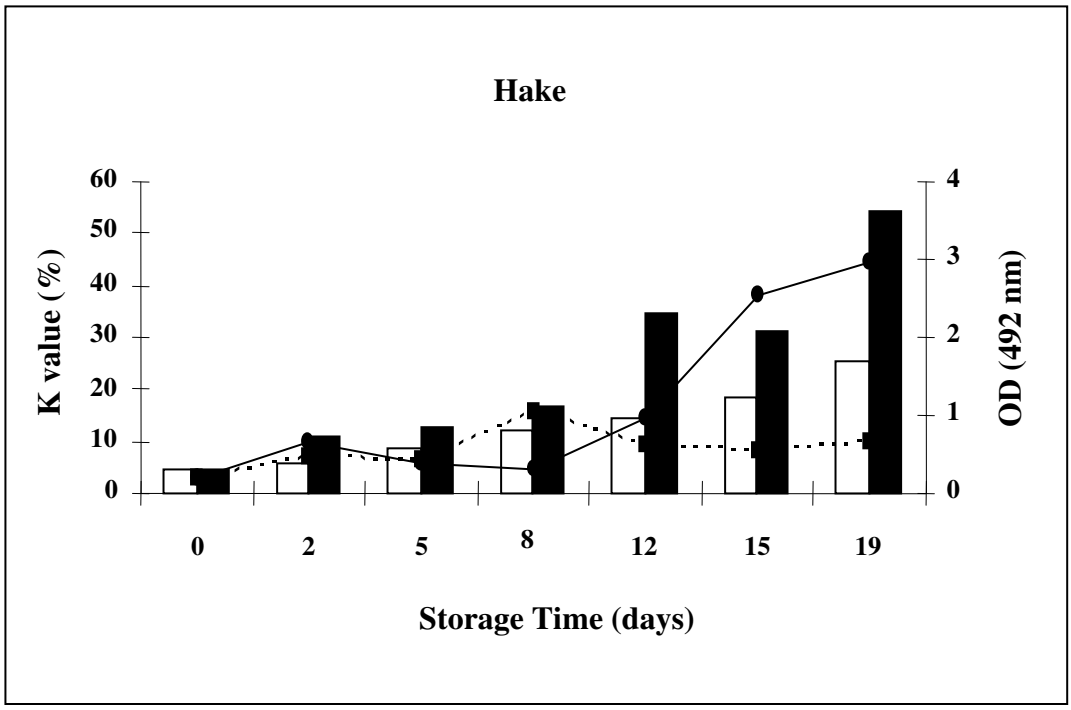


Figure *2ii*

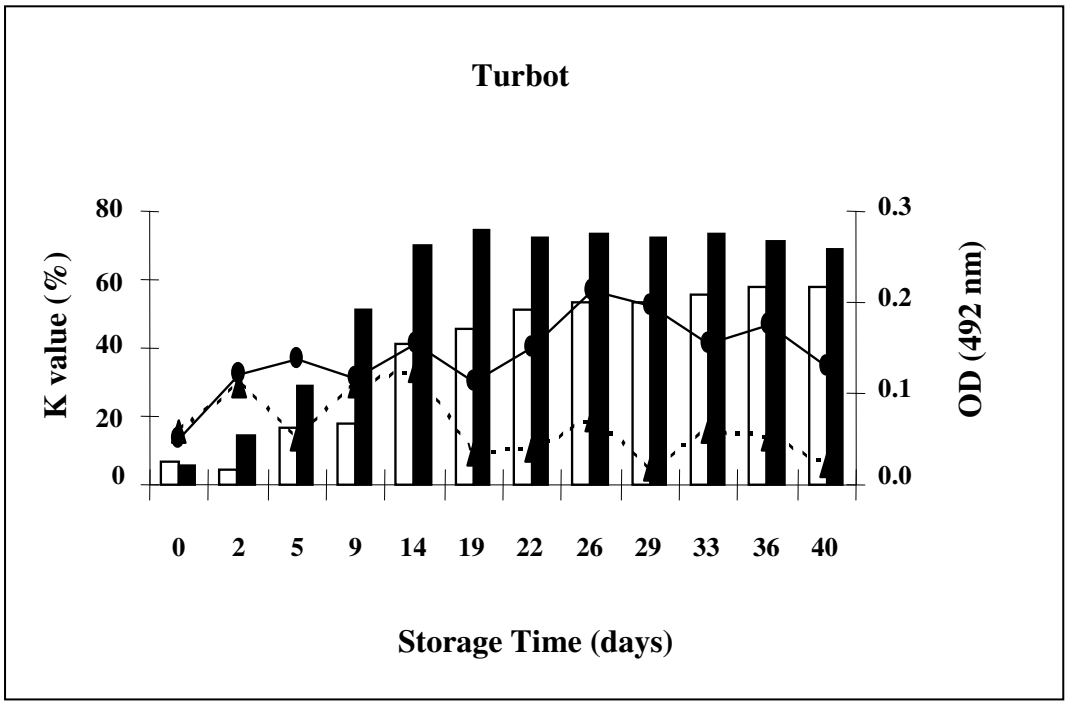


Figure 3

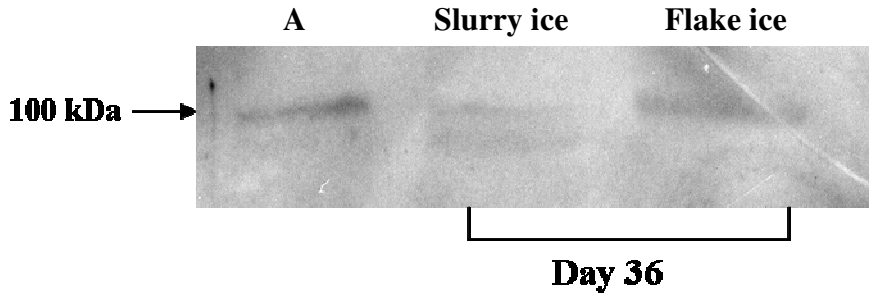
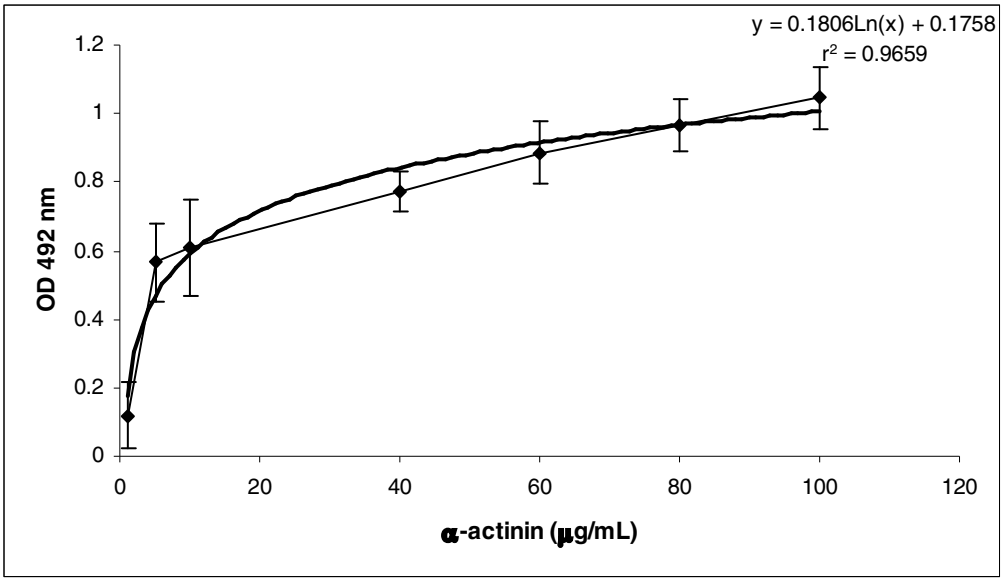


Figure 4


y = 0.1806Ln(x) + 0.1758
r² = 0.9659
OD 492 nm
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
α-actinin (μg/mL)